United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 902

Supplemental Assay Method for Testing Growth-Promoting Qualities of Brain Heart Infusion Agar using *Bacillus subtilis* Spores and *Candida krusei* as Indicator Organisms

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1. Introduction

This is a Supplemental Assay Method (SAM) for testing Brain Heart Infusion Agar (BHIA) for growth promoting qualities, as required in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.25(b). Each lot of media that is used in sterility tests (9 CFR 113.26 – 113.27) must be tested to ensure that it will support the growth of contaminants, should they be present in the biologics sample being tested for sterility. BHIA is one of the media that is used in codified sterility tests.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 30º- 35ºC incubator

2.1.2 20º- 25ºC incubator

2.1.3 Sterile disposable cotton-plugged pipettes

2.1.4 Sterile 10-mL disposable syringes with needles

2.1.5 Petri dishes, 15 x 100-mm, sterile

2.1.6 Class II biosafety cabinet

2.1.7 Vortex mixer

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Indicator Organisms: Use *Bacillus subtilis* (American Type Culture Collection [ATCC] #6633) and *Candida krusei (*ATCC #6258) or equivalent organisms as specified in the current United States Pharmacopoeia

2.2.2 Media: Brain-Heart Infusion Agar (BHIA), with 500 Kinetic (Kersey) units of penicillinase per mL of media--National Veterinary Services Laboratories (NVSL) Media #10204 (250-mL aliquots in 500-mL screw-capped flasks);

Soybean-Casein Digest Medium (SCDM)--NVSL Media #10423 (9-mL aliquots in 16 x 125-mm screw-capped tubes)

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Turn on biosafety cabinets at least 30 minutes before preparing positive control reagents or testing media for growth promotion.

3.2.2 Monitor incubators, freezers, and coolers daily for temperature.

3.3 Preparation of *Bacillus subtilis* indicator organism

Prepare this according to the current version of **SAM 900, Section 3.3**.

3.4 Preparation of *Candida krusei* indicator organism

Prepare this according to the current version of **SAM 900, Section 3.4**.

4. Performance of the Test

4.1 Establishing the working dilution of the indicator organisms

Titrate each new lot of indicator organism to determine the optimum working dilution. This working dilution will be used to test new batches or lots of media for growth promoting qualities.

4.1.1 Remove a vial of the newly prepared stock culture from the freezer and rapidly thaw. If the culture is lyophilized, rehydrate it with 1 mL of SCDM.

4.1.2 Make tenfold dilutions of the stock cultures. Use a 1-mL pipette to deposit 1 mL of the stock culture in 9 mL of SCDM.

4.1.3 Mix by inverting or vortexing the tube.

4.1.4 Using a pipette, transfer 1 mL of the diluted culture into another tube containing 9 mL of SCDM. Mix as before and continue the procedure until 10^{-10} dilution is prepared.

4.1.5 Incubate the *B. subtilis* dilution tubes for 24 to 48 hours at 30°- 35°C. Incubate the *C. krusei* dilution tubes for 2 weeks at 20°- 25°C.

4.1.6 Examine the tubes visually for growth to establish the growth endpoint (the highest dilution with observable growth) of the stock culture.

4.1.7 Prepare a new series of tenfold dilutions with a second vial of stock culture. Prepare dilutions to within 1 dilution of the growth endpoint (**Section 4.1.6**). For each of the last 3 dilutions, deposit 0.3 mL onto each of 2 sterile 15 x 100-mm petri dishes.

4.1.8 Pour 15-25 mL of Brain Heart Infusion Agar, with penicillinase, into each plate and swirl the plates to dispense the organisms. After the plates have solidified, incubate those containing *B. subtilis* for 7 days at 30°- 35°C. Incubate the plates containing *C. krusei* for 14 days at 20°- 25°C.

4.1.9 Count the number of colonies on all plates after the appropriate incubation period.

4.1.10 Pick the dilution ("working dilution") and the amount of inoculum (0.1-0.5 mL) which gives an average plate count of 20-60 colony forming units (CFU).

4.1.11 Due to a variation between batches of media and between vials of the standard organisms, a statistical tolerance is needed. The tolerances listed in **Appendix III** give the control limits for the average plate count which is found. To collect this data to determine the statistical tolerance, repeat **Sections 4.1.7 through 4.1.10** with 10 different vials of stock culture. Plate each vial onto 10 different batches of acceptable media (i.e., batches that have already tested satisfactorily for growth promotion).

4.2 Testing new lots of media for growth promotion

4.2.1 Test each lot of media with *C. krusei* and *B. subtilis* indicator organisms. Make tenfold dilutions (1 mL into 9 mL) of each indicator organism in SCDM to reach the working dilution for the organism lot, as established in **Section 4.1**.

4.2.2 For each indicator organism, deposit into each of two sterile 15 x100-mm petri dishes the volume of inoculum determined in **Section 4.1** that will yield 20-60 CFU.

4.2.3 Pour 15-25 mL of the new lot of BHIA with penicillinase into each of the 4 plates.

4.2.4 Incubate the 2 petri dishes containing the *C. krusei* culture at 20°- 25°C and count the number of colonies at the end of a 14-day incubation period.

4.2.5 Incubate the 2 petri dishes containing *B. subtilis* culture at 30°- 35°C and count the number of colonies at the end of a 7-day incubation period.

5. Interpretation of the Test Results

The average colony count per plate is determined for each organism and is compared with the control limits in **Appendix III** to see if the average falls within the limits expected. If the average colony count is not within the control limits, the growth-promoting quality of that batch of media is in question and all tests with satisfactory (SAT) results will be reported as no tests (NT). All tests with unsatisfactory (UNSAT) results, when the growth-promoting qualities of the media are in question, will be repeated using a new batch of BHIA media. If after repeating the test and the media's growth promoting qualities are still in question, the media must not be used and all tests already conducted with this media must be considered NT.

6. Report of Test Results

Report results of the test(s) as described by standard operating procedures.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.25, U.S. Government Printing Office, Washington, DC.

7.2 The U.S. Pharmacopoeia, 1985, Vol. 21, pp 1151-1160, Mack Publishing Co., Easton, PA.

8. Summary of Revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- The document number has been changed from STSAM0902 to SAM 902.
- The Contact has been changed from Gerald Christianson to Sophia G. Campbell.
- **1:** Information has been added to clarify the testing purpose.
- **2.1.5:** Petri dishes have been added to the equipment list.
- **2.1.6:** The class of biosafety cabinet to be used has been added.
- **2.2:** The list of reagents/supplies has been updated.
- **3.1:** Personnel qualifications have been clarified
- **3.2.2:** This section has been revised to indicate additional equipment monitored.
- **4.1/4.2:** These sections have been revised to clarify the procedures followed in testing.
- **5:** The test interpretations have been clarified.
- **Appendices I & II:** Media storage conditions have been added.
- The statistical tolerances table has been moved from the body of the document to **Appendix III**.

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Appendices

Media Formulations

Appendix I

Brain Heart Infusion Agar (BHIA)--NVSL Media #10204

Brain Heart Infusion Agar	52 g
QH_2O	1000 mL

Autoclave 20 minutes at 121ºC. Store at 2°- 5°C for no longer than 3 months.

Appendix II

Trypticase Soy Broth (TSB) or Soybean-Casein Digest Medium (SCDM)--NVSL Media #10423

Trypticase Soy Broth	30 g
QH_2O	1000 mL

Autoclave 20 minutes at 121ºC. Store at 20°- 25°C for no longer than 3 months.

TSB and SCDM are 2 names for the same media formulation from different media companies.

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Appendix III

Statistical Tolerances
Control Limits for Single Batch of Media -- Average of 2 Plate Counts

Average Plate Count in Preliminary Test	Control Limits *Bacillus*	*Candida*
20	6 - 34	8 - 32
25	9 - 41	12 - 38
30	12 - 48	16 - 44
35	15 - 55	20 - 50
40	18 - 62	24 - 56
45	20 - 70	28 - 62
50	22 - 78	32 - 68
55	25 - 85	36 - 74
60	28 - 92	40 - 80